(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 October 2001 (11.10.2001)

PCT

(10) International Publication Number
WO 01/74990 A1

(51) International Patent Classification[7]: C12M 1/00

(21) International Application Number: PCT/NL01/00273

(22) International Filing Date: 3 April 2001 (03.04.2001)

(25) Filing Language: Dutch

(26) Publication Language: English

(30) Priority Data:
1014825 3 April 2000 (03.04.2000) NL

(71) Applicant *(for all designated States except US)*: **STICHTING ENERGIEONDERZOEK CENTRUM NEDERLAND** [NL/NL]; Westerduinweg 3, NL-1755 LE Petten (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **REITH, Johannes, Henricus** [NL/NL]; Burmanstraat 25-3, NL-1091 SH Amsterdam (NL). **HACK, Cornelis, Johannes, Josef, Marianus** [NL/NL]; Bolbaan 3, NL-4751 RL Oud Gastel (NL). **KALWIJ, Ronald** [NL/NL]; Venkelhof 43, NL-4907 HH Oosterhout (NL). **MUR, Lucas, Roelof** [NL/NL]; Demmerik 21, NL-3645 EA Vinkeveen (NL).

(74) Agent: **JORRITSMA, Ruurd**; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

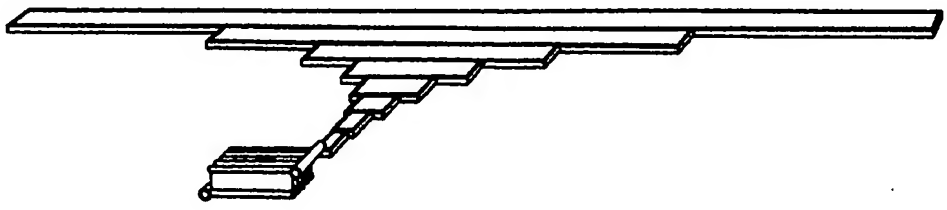
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS OF CULTURING ALGAE



(57) Abstract: Algae and other photosynthesising micro-organisms can be expediently cultured in an open system by inoculating an aqueous medium with one or more species or strains from the said group of micro-organisms, culturing the micro-organisms and recovering the biomass produced. According to the invention, the photosynthesising micro-organisms should be cultured in a series of open reactors of which the first is inoculated with a large quantity of inoculum which has been precultured in a closed photobioreactor.

WO 01/74990 A1

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Martin E. Hsia 32,471

Date: 9/21/08

Process of culturing algae

The invention relates to a process of culturing photosynthetic micro-organisms (comprising the groups of micro-algae and cyanobacteria) on an industrial scale. The invention is explained here in more detail with reference to algae as representatives of the photosynthetic micro-organisms. Important taxonomic groups in the algae world include: green algae, red micro-algae, diatoms, dinoflagellates.

Algae cultivation is an environmentally friendly and energetically efficient process for the production of organic material by photosynthesis from carbon dioxide and luminous energy. In this process, use is made of gratis energy from sunlight, gratis carbon dioxide and water, which can be of low quality, including industrial process water, effluent of biological water treatment or other waste water streams. Products of algae cultivation include algal biomass and purified water which can be used, for example, as industrial water. If the carbon dioxide stems from flue gas, this algae production also contributes to flue gas cleaning, not least because nitrogen compounds (NOx) too can be removed from the flue gas by the algae.

The algal biomass produced can - depending on the cultured species - be used in the extraction of a series of high-value-added substances such as: fatty acids (including polyunsaturated fatty acids), pigments, polysaccharides, and a number of other biologically active substances. These products can be used in nutrition and nutritional supplements, cosmetics and other "personal care" products and in clinical and pharmaceutical products. The remainder of the biomass can be designated as animal feed, fertiliser or as a raw material for energy production. Integration of production functions and environmental functions is a beneficial feature of algae cultivation.

Culturing algae requires the input of (sun)light (as an energy source for photosynthesis) and a sufficient supply of nutrients in dissolved form in the culture medium. In particular, these are: carbon in the form of $ÇO_2$, HCO_3^- or CO_2 derived from mineralisation of organic substances in the feed water, a nitrogen source (generally NO_3^-, NH_4^+ or urea), phosphate and a number of other nutrients including sulphur, potassium, magnesium and trace elements. If waste water is used for culturing, this - depending on the composition - requires the addition of nutrients to ensure that enough of all the necessary nutrients is supplied. The necessary addition of nutrients depends on the type of waste water. Effluent of biological water purification may, for example, require phosphate and trace elements to be added to enable good growth. Effluent or other waste water generally (also) contains carbon in bound form (COD). It was found that this

organic substance in the algae system is mineralised by bacteria, thus making CO_2 available for uptake by the algae. In addition, a number of photosynthetic micro-organisms are capable of taking up organic substances directly and using them as a nutrient and an energy source, in combination with the photosynthetic process. Alternatively "clean" culturing is also possible by making use of, for example, surface water or tap water, to which the nutrients are added in the form of e.g. artificial fertiliser products and pure, technical-grade CO_2.

In practice up to now, two types of algae culture systems are in use. On the one hand, there are the open systems comprising open, shallow ponds with a mixing arrangement, usually of annular design (e.g. "high rate algal pond", HRAP). On the other hand there are the closed photobioreactor systems which usually take the form of upright or horizontal tube or panel systems.

The open systems, in order to increase their efficiency, are generally designed as a continuous culture in which a fixed supply of culture medium or influent ensures constant dilution of the system. The organisms adapt their growth rate to this dilution regime, the organism best adapted to the environment prevailing in the system winning the competition with the other organisms.

A drawback of the common open algae culture systems is the major risk of infection by undesirable photosynthetic micro-organisms which can be introduced via air or rain. In practice it is therefore not possible in open systems to successfully culture an alga species chosen at will, since algae which infect the system (often green algae) will generally soon predominate in the system, thereby upsetting the composition of the algal biomass. As a result, the composition and quality of the biomass produced (and thus the yield of the desired product) cannot be controlled. Such infections can be prevented only by choosing a culture medium which is unfavourable for infectious and other undesirable micro-organisms and favourable to growth of the desired alga species, so that the latter can win the competition. In a limited number of cases this is possible. For example, in the open culture of *Spirulina* a high pH and high alkalinity (acid-absorbing capacity) are selective for this alga species. Apart from *Spirulina* a number of *Chlorella* species (such as *C. pyrenoidosa* and *C. vulgaris*) and *Dunaliella* species (inter alia *Dunaliella salina*) are thus grown on a relatively large scale. The selective advantages which make this possible for these groups of algae are, respectively: the high growth rate which allows the competition from other organisms to be won (in the case of *Chlorella sp.*) and the salt water environment (in the case of *Dunaliella sp.*). For most micro-algae species, however, it is the case that the culture conditions are insufficiently selective to enable

readily controllable cultivation in large-scale open systems. Consequently, the production potential of algae (according to one estimate, 30,000 species exist) remains largely unused. Even if the application of selective conditions via the composition of the culture medium is possible, this has drawbacks, as the effluent after separation of the biomass cannot be reused. In the cultivation of *Spirulina sp.*, for example, the consequence is that the effluent has a high pH and alkalinity and thus is unusable for many purposes. While cultivation in saltwater - as with *Dunaliella sp.* - is selective for *Dunaliella* (and possibly other algae groups) it equally does not lead to a reusable effluent. Culturing selected algae species in an open continuous culture in combination with reuse of the effluent has consequently until now been entirely impossible.

An alternative to the outlined problems could be to carry out algae cultivation in closed photobioreactors. In these, the process conditions can be accurately controlled, and no infections carrying undesirably alga species will occur. A major drawback of the closed photobioreactors resides in the high investment costs which lead to high production costs. In addition, the technology of the photobioreactors is as yet not sufficiently developed for large-scale application.

An algae culture system has been found now, where high production can go hand in hand with a high degree of purity of the alga species chosen, i.e. with minimal contamination with other, undesirable species, and from which, after separation of the algae produced from the liquor, an effluent results which can be usefully employed, e.g. as industrial water. The process according to the invention is described in more detail in the accompanying claims and in the following explanation.

The process of culturing algae according to the invention is based on the use of a series of open reactors connected in series. The total system behaves as a plug-flow reactor, increasingly so as the number of connected reactors increases. This plug-flow reactor is inoculated, preferably continuously or alternatively periodically, with a large quantity of algae which have been precultured under controlled conditions in a closed photobioreactor and/or some other culture system sealed off from the outside air or adequately covered (e.g. a greenhouse). A closed reactor here therefore refers to a reactor which is adequately sealed from possible sources of infection; depending on circumstances, this can also be a semi-closed reactor.

The large quantity of algae with which the plug-flow reactor is inoculated from the closed preculture is at least 10^5 cells per charge; on the other hand, this quantity can be expressed in terms of the ultimately recovered quantity of algae or photosynthetic bacteria, preferably at least 100 ppm of that ultimate quantity.

A characteristic of the reactor system of the plug-flow type is that no macro-back-mixing takes place, thus ensuring that any contamination which may occur cannot develop to higher densities and will be flushed out. The underlying principle of bulk inoculation with a controlled preculture is illustrated within the following frame.

The growth of algae

If the generation rate of an alga equals T, the biomass after a period t can be calculated by the following equation

$$N_t = N_0 \, 2^{t/T}$$

(N_0 is biomass at t = 0)

The biomass formed after a period t is therefore defined by N_0 and the generation time T

Example:

If inoculation involves 10^6 cells of species A having a generation time of 24 hours, the biomass after 240 hours will be:

$$N_{240} = 1.024 \, . \, 10^9 \text{ cells}$$

An infection with species B which enters at the start of the system and has a generation time which is half that of organism A will, after 240 hours, have a biomass of:

$$N_{240} = 1.049 \, . \, 10^6 \text{ cells}$$

Conclusion

Even though the growth rate of the infection is twice as high, it only forms 0.1% of the biomass after 10 generations. This is caused by the bulk initial inoculation with species A (10^6 cells) compared with the initial inoculation of the infection (1 cell).

A open system based on bulk inoculation and a constant stream in one direction can be used to produce relatively slow-growing alga species without problems with aerial infections. In principle, it is thus possible to culture any alga species desired.

The series of connected open reactors numbers at least three reactors, preferably at least four reactors. The connection is such that as the contents of the one reactor are transferred to the next reactor, not more than 5% backmixing takes place, i.e. that not more than 5% of the contents of the reactor which are passed on to the downstream reactor are mixed with the next contents, from the upstream reactor. Preferably, this percentage is lower, for example at most 2% or rather at most 1%. It will be evident that the lower this backmixing percentage, the smaller the series of reactors which is sufficient with regard to restricting infections.

A conceivable design of such a system involves a cascade of connected tubular or trough reactors (troughs, panels, tubes or comparable) or a cascade of ideal stirred reactor systems (CISRTs). The latter can be either ideal stirred tank reactors or tubular reactors comprising static mixers or equivalent systems.

In the connected elements of the system, an exponential increase in the biomass takes place (as explained within the frame above). Additionally it is important, in order to achieve the highest possible algal productivity, to maintain the biomass density in the system as accurately as possible at an optimal, constant value, in order to allow the conversion of the incident light into algal biomass to proceed as efficiently as possible. This optimum density is a function of the incident light intensity and the depth of the culture. As the biomass density must remain optimal and the water depth must not increase, the elements of the system will preferably show an exponential increase in surface area. The increase in surface area can be obtained by an increase in the number of elements in the downstream direction. Instead, an increase in surface area can be obtained by an increase in the reactor volume in the downstream direction. The volume increase is achieved by additional culture medium or waste water containing nutrients being fed in at two or more locations, thereby maintaining the algae concentration at a constant level. This admixture is quantified in terms of the algae concentration and consequently of the growth rate of the algae in the system. Consequently, the flow velocity is likewise a function of the instantaneous growth rate of the cultured alga. At the end of the system, the algae are separated from the culture medium, thereby affording as products: purified water and algal biomass as a raw material for the extraction of products.

Figure 1 illustrates the principle with reference to a system consisting of a closed tubular bioreactor for continuous inoculation of the open system section, which - in this example - consists of open troughs.

The system is suitable, in principle, for large-scale culture of all desired species and strains of photosynthetic micro-organisms, including those species and strains which in the present state of the art can be cultured successfully only in closed reactor systems, because of the above-described contamination problems. This ensures that - by virtue of the "free" choice of species and/or strains - it is also possible to make a "free" choice of the desired products from the range of components which are produced by photo-synthetic micro-organisms, and that considerably lower production costs per unit of biomass and per unit of product are achieved, as the system found requires considerably lower investment than closed bioreactor systems on a comparable scale.

The process and the system according to the invention are suitable for any species or strain from the group of photosynthetic micro-organisms. This comprises photosynthetic bacteria and micro-algae from the order Cyanobacteria (formerly sometimes also referred to as algae order Cyanophyta), the order Chlorophyta (green algae), the order Chromophyta, the order Cryptophyta, the order Pyrrhophyta (dinoflagellates), the order Euglenophyta and the order Rhodophyta (red micro-algae). The Chromophyta, according to this classification, inter alia include the classes Bacillariophyceae (diatoms), Chrysophyceae (golden algae), Eustigmatophyceae and Xanthophyceae (yellow-green algae). According to older classifications, some of these classes form orders of their own (Bacillariophyta, Xanthophyta and the like).

Examples are the culture of *Monodus* species (Eustimatophyceae) for the production of polyunsaturated fatty acids. To be mentioned among these is the species *Monodus subterraneus*, a freshwater species having an optimum culture temperature of about 25°C and an optimum growth rate (μ) of about 0.04 h^{-1}. Other examples are species and strains from the micro-algae genera *Porphyridium sp.* (Rhodophyta) for the production of phycobiliproteins and *Chlorella sp.* (Chlorophyta) for the production of carotenoids and bioactive substances, and the cyanobacteria genera *Nostoc sp.*, (for the production of phycobiliproteins) and *Calothrix sp.* (for phycobiliproteins and by-products).

Description of the Figures:

Figure 1 gives an overview of an integral culture system according to the invention.

Figure 2 is a schematic depiction of the principle of a combination of a closed photobioreactor for producing the inoculum (A) and the open system section, the Multiplier (B) in which the algae reside for a number of generations (in this example 4 generations). The bottom section of the figure shows the principle of the open system section in which the surface area increases exponentially, as indicated by the exponential increase in the number of boxes. In practical implementation, these boxes will consist either of (connected) tubular or trough reactors (troughs, channels, tubes or the like) or (connected) ideal stirred reactor systems (CISTRs). On technical and economic grounds, these individual elements of the system will preferably have a larger surface area than the boxes as drawn, on within the condition that the surface area of the system as a whole increases exponentially in the direction of the plug flow.

CLAIMS

1. A process of culturing photosynthetic micro-organisms in an open system by inoculating an aqueous medium with one or more species or strains from the group of the photosynthetic micro-organisms, culturing the micro-organisms and recovering the biomass produced, characterised in that the photosynthetic micro-organisms are cultured in a series of open reactors of which the first is inoculated with inoculum which has been precultured in a closed photobioreactor.

2. A process according to Claim 1, wherein the algae are cultured in the series of open reactors over from 3 to 15 generations.

3. A process according to Claim 1 or 2, wherein the biomass concentration in the series of open reactors is kept essentially constant.

4. A process according to any one of Claims 1-3, wherein the surface area of the series of open reactors doubles in the downstream direction for every generation or every two generations of the cultured organism.

5. A process according to any one of Claims 1-4, wherein medium is added at two or more locations in the series of open reactors.

6. A process according to any one of Claims 1-5, wherein the backmixing of reactor contents of an open reactor towards the open reactor located directly upstream amounts to at most 5%.

7. A process according to any one of Claims 1-6, wherein the medium used comprises waste (water) streams, with or without the provision of supplementary nutrients.

8. A process according to any one of Claims 1-7, wherein the resulting effluent, after the biomass has been separated off, is used, optionally following one or more supplementary purification steps, to be employed as industrial water.

9. A process according to any one of Claims 1-8, wherein photosynthetic micro-organisms from the group of micro-algae are cultured.

10. A process according to Claim 9, wherein photosynthetic micro-organisms from the group of Chlorophyta (green algae) or Chromophyta are cultured.

11. A process according to Claim 9, wherein photosynthetic micro-organisms from the group of Cryptophyta (golden algae) or Pyrrhophyta (dinoflagellates) are cultured.

12. A process according to Claim 9, wherein photosynthetic micro-organisms from the group of Euglenophyta or Rhodophyta are cultured.

13. A process according to any one of Claims 1-9 wherein photosynthetic micro-organisms from the group of cyanobacteria are cultured.

Fig 1

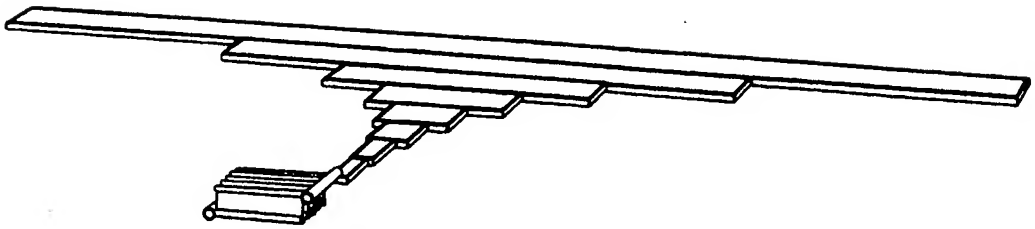
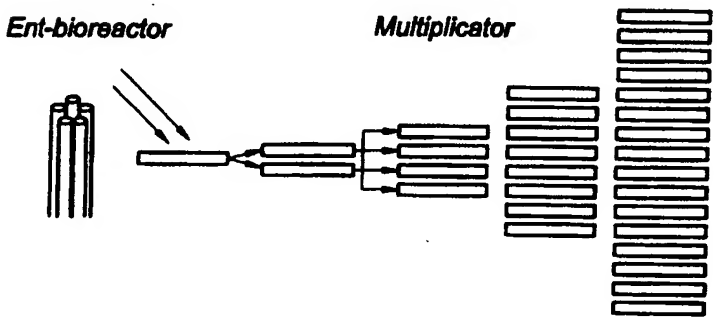


Fig 2



INTERNATIONAL SEARCH REPORT

Internati pplication No
PCT/NL 01/00273

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M A016

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 43 17 006 A (VIEH & FLEISCH GMBH) 24 November 1994 (1994-11-24) claim 1	1
A	FR 1 579 556 A (CITY OF KIRYU) 29 August 1969 (1969-08-29) claim 1	1
A	US 4 065 875 A (SRNA RICHARD FRANK) 3 January 1978 (1978-01-03) column 2, line 22 - line 35; figure 1	
A	US 3 763 824 A (SCHOON D) 9 October 1973 (1973-10-09)	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2001	11/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Coucke, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International publication No
PCT/NL 01/00273

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4317006 A	24-11-1994	NONE	
FR 1579556 A	29-08-1969	DE 1767195 A	14-10-1971
		GB 1214701 A	02-12-1970
		US 3546812 A	15-12-1970
US 4065875 A	03-01-1978	NONE	
US 3763824 A	09-10-1973	NONE	